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Pinocytosis in Ehrlich Tumor Cells: Quantitative Determination and Regulation

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SUMMARY

A method for the quantitative determination of pinocytosis in Ehrlich ascites tumor cells was developed using iodinated concanavalin A as a probe. Substantiating data were presented as proof of the validity of employing α-methyl mannoside-resistant incorporation into cell pellets as a means of measuring pinocytosis. By this method, factors that influenced the rate of pinocytosis were studied, and temperature dependency, time coures of the reaction, ATP requirement for the pinocytotic process in the tumor cells were reported. Insensitivity of pinocytosis in the tumor cells to cytoskeletal inhibitors, *i. e.* cytochalasin D and colchicine, was discussed in comparison to the other membrane related processes previously studied by our group, such as HVJ-induced cell fusion and phagocytosis.

INTRODUCTION

Pinocytosis has been studied extensively by morphorogical (electron microscopical) techniques. From these studies it can be concluded that at least two different routes of pinocytosis are present; one of which utilized coated pits and another, via smooth (non-coated) membrane invagination is present in several types of cells (6, 7, 9, 20). From the difference in sensitivity of these pinocytotic processes to several influencing factors, it has been suggested that different regulatory mechanisms may be operating on these processes (5, 12, 18). But, it seems rather difficult to drow a simple conclusion on the regulatory mechanism of pinocytosis from data published to date, since property of pinocytosis of a single types of cells could be readily modified by changing the condition of these cells. For example, the basal rate of pinocytosis of peroxidase by macrophage is inhibited only marginally by cytochalasin B, whereas that of phorbol myristate acetate-stimulated

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macrophage is decreased by more than 50% by the same microfilament inhibitor (17). Thus, quantitative studies on pinocytosis of different types of cells may be required for the full understanding of mechanism of pinocytosis.

Dynamic changes of plasma membrane during several cell surface-related phenomena including phagocytosis, pinocytosis, cell fusion and cap formation may be regulated by cytoskeleton in a different mannor. Therefore, for obtaining a comprehensive view of the membrane-cytoskeleton interaction, it may be necessary to study effect of several cytoskeletal inhibitors and other inhibitors on these membrane-related phenomena using the same cell. Since we have found previously that HVJ (Sendai virus)-induced fusion of Ehrlich ascites tumor cells was almost completely inhibited by cytochalasin B and D (2), we attempted to study the regulation of pinocytosis using the same cancer cells. Thus, the method of quantitation and effects of inhibitors on pinocytosis of concanavalin A in Ehrlich tumor cell will be described in this report.

MATERIALS AND METHODS

Cells and virus

An 8-azaguanine-resistant strain of Ehrlich ascites tumor cells was propagated in the peritoneal cavities of ddN mice. The cells were freed from contaminating erythrocytes and leukocytes by washing three times with a balanced salt solution consisting of 140 mM NaCl, 54 mM KCl, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4 buffered with 10 mM Tris-HCl at pH 7.6 (BSS). The washed cells were suspended in BSS. The cell number was measured by Fuchs-Rosenthal Cytometer after appropriate dilution. HVJ (Sendai virus), Z strain, was grown in embryonated eggs, purified by differential centrifugation, and suspended in BSS (15). The dose of the virus was expressed in terms of its hemagglutination units (HAU), which was determined by Salk's pattern method (19).

Materials

Concanavalin A (con A) was purchased from Boehringer Mannheim, ferritin (type I), horse raddish peroxidase (type II) was obtained from Sigma, and carrier free iodine (IMS 30) was purchased from Radiochemical Centre Amersham. Cytochalasin D was kindly provided by Dr. Minato of Sionogi Phermaceutical Co., Osaka. FCCP (carbonyl cyanide *p*-fluoromethoxyphenylhydrazone) was a gift from Dr. P. G. Heytler of du Pont.

Pinocytosis of concanavalin A

Con A (5 mg) dissolved in 4 *ml* of 50 mM potassium phosphate buffer (pH 7.0) was mixed with carrier free iodine to give a final ^{125}I concentration of 25 μCi/*ml*. Chloramine T (100 μg) dissolved in 0.1 *ml* of the same buffer was added to the above mixture at 4°C, and iodination reaction proceeded on ice for 10 min. The

reaction was terminated by the addition of 100 μg sodium metabisulfite dissolved in 0.1 *ml* of the same buffer, and unbound iodine was removed by dialysis against the same buffer with several changes of the buffer.

Reaction mixture for pinocytosis consisted of 0.2 *ml* of 10% suspension of Ehrlich tumor cells, 28 μg to 40 μg of con A containing 20,000 to 100,000 cpm of ^{125}I, and total volume was adjusted to 1 *ml* with BSS. After preincubation at 0° C for 5 to 10 min as indicated, pinocytosis was started by increasing temperature to 30°C with gentle shaking. Reaction was terminated after 30 min by the addition of ice cold BSS (either containing 50 mM α-methylmannoside or without it), and centrifuged at 2,000 rpm for 15 min in a cold room (4°C). Washing by the same medium was repeated two times, and resultant cell pellets were counted with a gamma counter. Preincubation of the cells with inhibitors was performed in BSS for 30 min at 30°C before the addition of con A, after cooling the preincubated mixture in an ice bath, con A was added to the system and the reaction was performed as described above. Cell fusion reaction was performed in BSS containing 2 mM $CaCl_2$ (BSS-Ca^{2+}) by addition of 1,000 HAU of HVJ as described previously (16) at 30°C for 15 min. The reaction was terminated by the additon of ice cold BSS-Ca^{2+} and fused cells were washed two times with BSS. Finally, fused cells were suspended in BSS to give the origial volume, and pinocytosis is performed as described above.

Electron microscopy

Ferritin-con A conjugates were prepared basically as described by Nicolson and Singer (13). Briefly, ferritin (63.5 mg) dissolved in 1 m*l* of 50 mM sodium phosphate buffer (pH 6.8) was mixed with con A (17.6 mg) and sodium chloride solution was added to give a final concentration of 0.2 M and the volume was adjusted to 2.4 m*l* by the phosphate buffer. Glutaraldehyde (0.5%) was added to give a final concentration of 0.05% and the mixture was gently stirred for 60 min at 25°C. After dialysis, aggregates were removed by centrifugation at 10,000 × g for 45 min and the resultant supernatant was fractionated by gel filtration with Biogel A 1.5 m column (3.0 × 77 cm) equilibrated with 50 mM sodium phosphate buffer (pH 6.8). Pinocytosis of ferritin-con A conjugates was performed as described for con A. Final pellets were fixed with 2.5% glutaraldehyde (dissolved in 100 mM sodium phosphate buffer (pH 7.4)) for 2 h at 0°C. After incubation with osmium tetroxide (1% in veronal-acetate buffer, pH 7.4), samples were embedded in Vestopal, thin sectioned, and stained by the usual procedure. Staining of con A by peroxidase-DAB procedure was carried out as described previously (3), based on the method of Bernhard and Avrameas (4).

RESULTS AND DISCUSSION

Pinocytosis of con A was measured as described in " Materials and Methods ". As shown in Fig. 1, binding of ^{125}I-labelled con A occurred at 0°C and increased slightly during the 5 min preincubation period. But, the total amount of cell-associated con A increased further (more than 2.5 fold) when temperature of incubation was shifted to 30°C. Specific binding of con A on cell surface, *i. e.* amount of the lectin which can be released by repeated washing with α-methylmannoside, did not increase appreciablly. Therefore, this increased binding can be attributed mostly to the internalization of the lectin into the cells, *i. e.* pinocytosis. Thus, we can quantitate pinocytosis of con A by measuring α-methylmannoside-resistant radioactivity remaining in the pelleted cells, although some contribution of non-specifically adsorbed lectin on the cell surface to the radioactivity could not be excluded. A method of estimation of non-specific binding will be described below. The rate of pinocytosis (plus non-specific binding) is dependent on the con A concentration as shown in Fig. 2, and is almost saturated at 30 μg.

If we assume that uncoupler-sensitive portion of incorporation of labelled

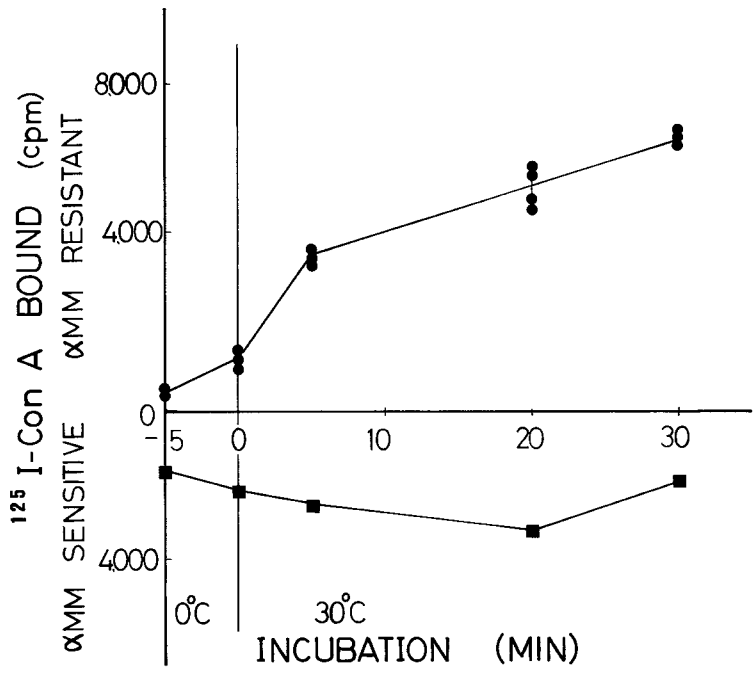

Fig. 1 Time course of α-methylmannoside sensitive binding and insensitive ones (pinocytosis) of concanavalin A to Ehrlich ascites tumor cells.

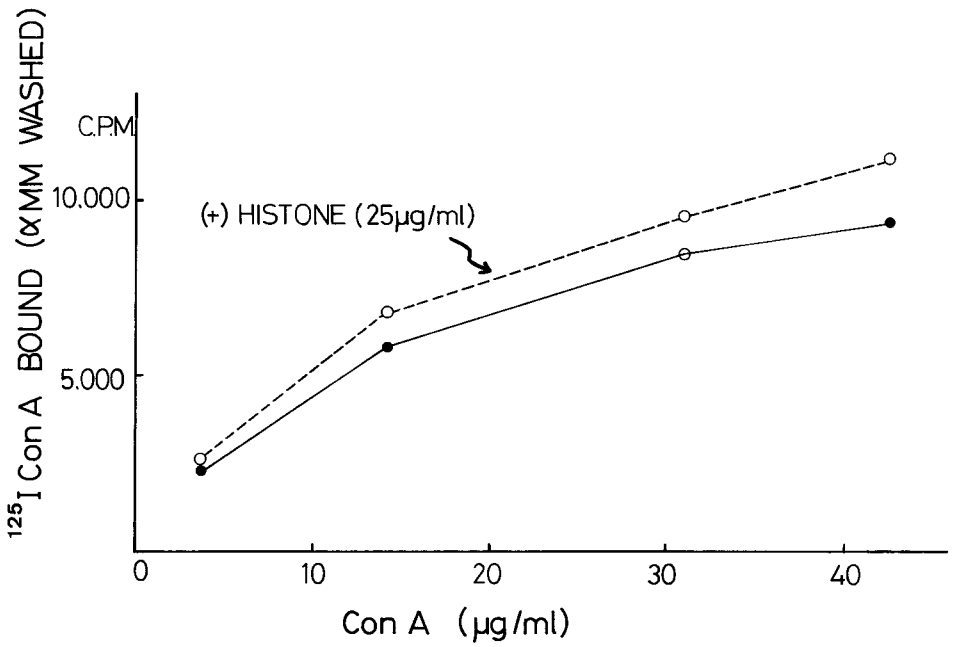

Fig. 2 Effects of concanavalin A concentration and addition of histone on pinocytosis (plus nonspecific binding) of the lectin in Ehrlich ascites tumor cells.

lectin to the cell pellets is a result of a type of pinocytosis, this process found to be temperature sensitive as is shown in Fig. 3. No such activity can be observed at 0° C and 10°C, while the activity increased extensively from 20 to 30°C (more than two fold). Since this concentration of FCCP was shown to deplete the ATP content of Ehrlich tumor cells by about 60% (1), dependency of pinocytosis on intracellular ATP concentration is strongly suggested. To confirm this conclusion further, the effect of different concentrations of two kind of uncouplers (dinitrophenol and FCCP) was studied (Fig. 4). The degree of inhibition of pinocytosis is almost identical with these uncouplers. To determine the contribution of glycolytic pathway for energy supply of pinocytosis, iodoacetamide was used as an inhibitor for this ATP-producing process, and it was found to be only partially inhibitory (Fig. 5). An addititon of iodoacetamide to FCCP-containing samples did not increase the effect of FCCP. Therefore, it may be said that intracellular ATP is decreased more by FCCP than iodoacetamide treatment, and the coexistence of FCCP and iodoacetamide may not further decrease the intracellular content of ATP. Ehrlich tumor cells fused to each other by HVJ exhibited very low, if any, pinocytotic activity. Since the ATP content during the cell fusion process decreased only marginally and transiently (15), this inhibition is not due to the

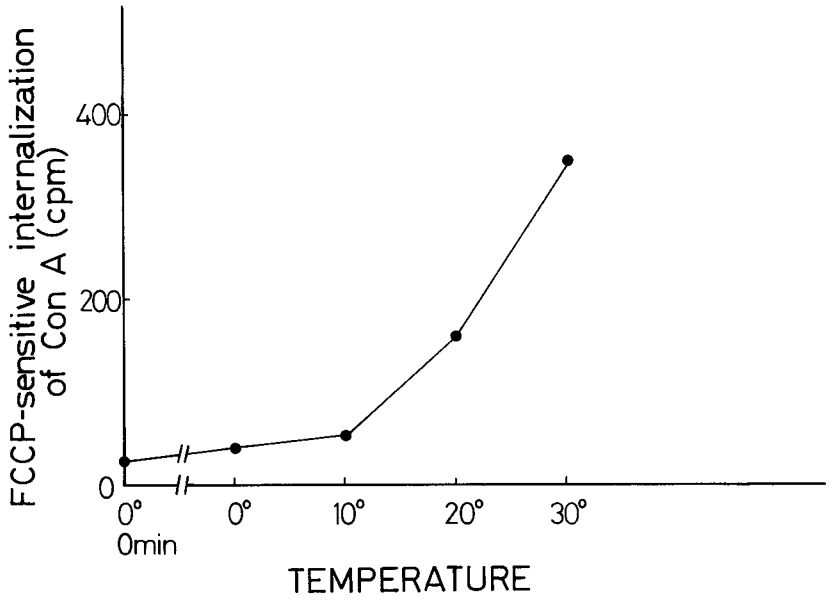

Fig. 3 Temperature dependency of FCCP-sensitive pinocytosis of concanavalin A.

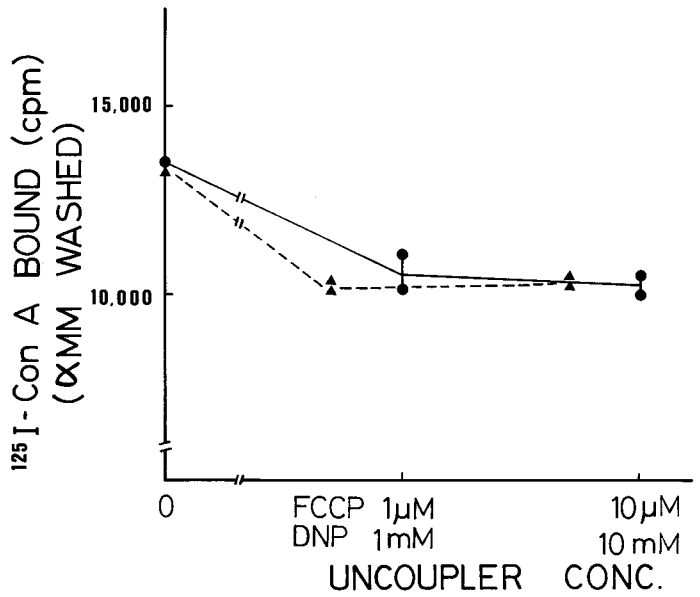

Fig. 4 Inhibition of concanavalin A pinocytosis by different concentrations of uncouplers, FCCP and dinitrophenol.

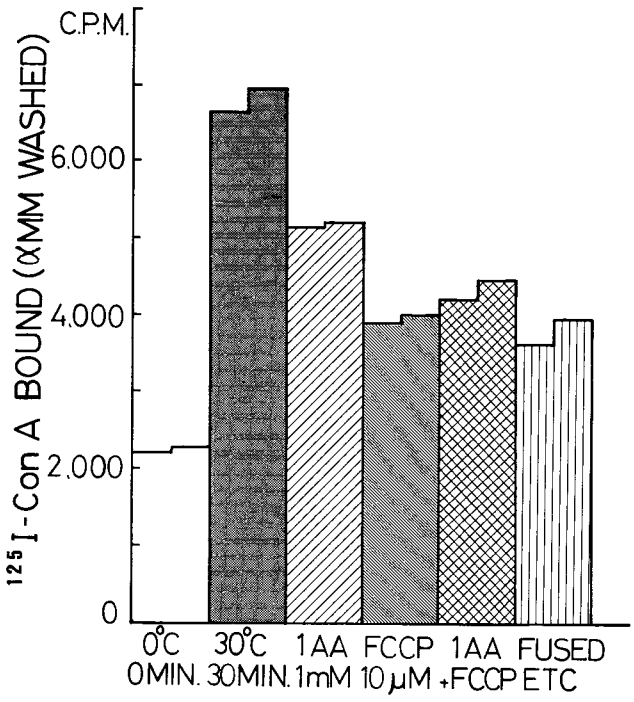

Fig. 5 Inhibition of concanavalin A pinocytosis by iodoacetamide, FCCP and combination of these inhibitors. ETC: Ehrlich tumor cells.

decrease of ATP in fused cells. It is interesting to note that the fused cells may have a quite different membrane-cytoskeleton interaction compared with the unfused cells, since the former cells have tendency to form a cap structure without addition of external ligands (14).

On the other hand, cytochalasin D (a microfilament inhibitor) at concentratitons completely inhibitory for HVJ-induced fusion (2) and actin-related gelation (11) of Ehrlich tumor cells, has no effect on pinocytosis (Fig. 6), indicating that the two plasma membrane-related process, *i. e.* cell fusion and pinocytosis, of Ehrlich tumor cells are controled differently. Colchicine, a well-known inhibitor of microtubules, is also not inhibitory even at a very high concentration (Fig. 7, a). Therefore, regulation of the pinocytotic process by known components of cytoskeleton is not substantiated, although intracellular ATP might exert its effect on pinocytosis through an unknown mechanism. Unlike pinocytosis, phagocytosis of mouse peritoneal macrophages, is known to be regulated cooperatively by microfilaments and microtubules (10). Extracellular calcium ion (Fig. 7, b) and intracellular cyclic AMP (Fig. 7, c) have no appreciable effect on pinocytosis,

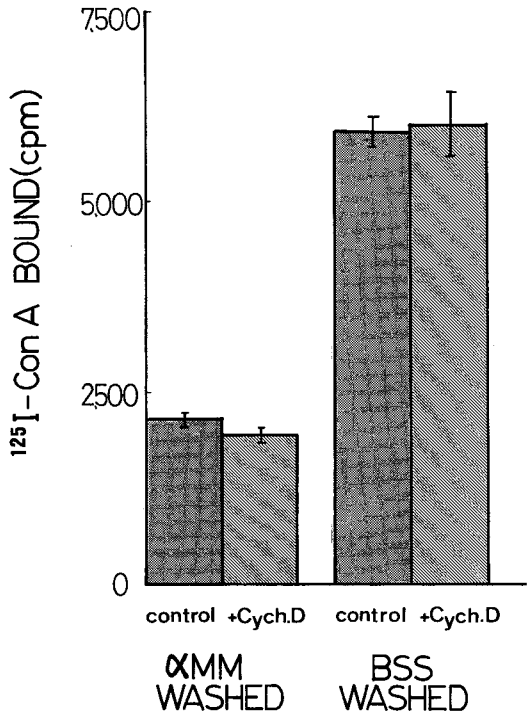

Fig. 6 Effect of cytochalasin D on nonspecific binding and pinocytosis of concanavalin A.

although both were shown to affect HVJ-induced cell fusion (15). Basic proteins, such as histone, are reported to stimulate pinocytosis in some cases (8), but are only slightly stimulatory in the case of con A pinocytosis by Ehrlich tumor cells (Fig. 2). This might mean that binding to and cross-linking of surface components by con A is by itself strong enough to support pinocytotic reaction.

Electron microscopic examination of pinocytosis

For confirmation of occurrence of pinocytosis under our experimental conditions, an electron microscopic examination was performed using ferritin -labelled con A as a probe. Ferritin-con A conjugates were prepared as described in " Materials and Methods ". As depicted in Fig. 8, the conjugates were fractionated by gel filtration using agarose (Biogel A 1.5 m) column for separation of the lectin-ferritin conjugates having different molecular ratios of the lectin to ferritin. Each fraction was iodinated as described for con A, and incorporation of ^{125}I-con A -ferritin conjugates was measured. As shown in Table I, fraction II which is assumed to contain 1 : 1 conjugates of ferritin and con A showed very similar results with con A in pinocytosis and non-specific binding to the cell surface, and therefore,

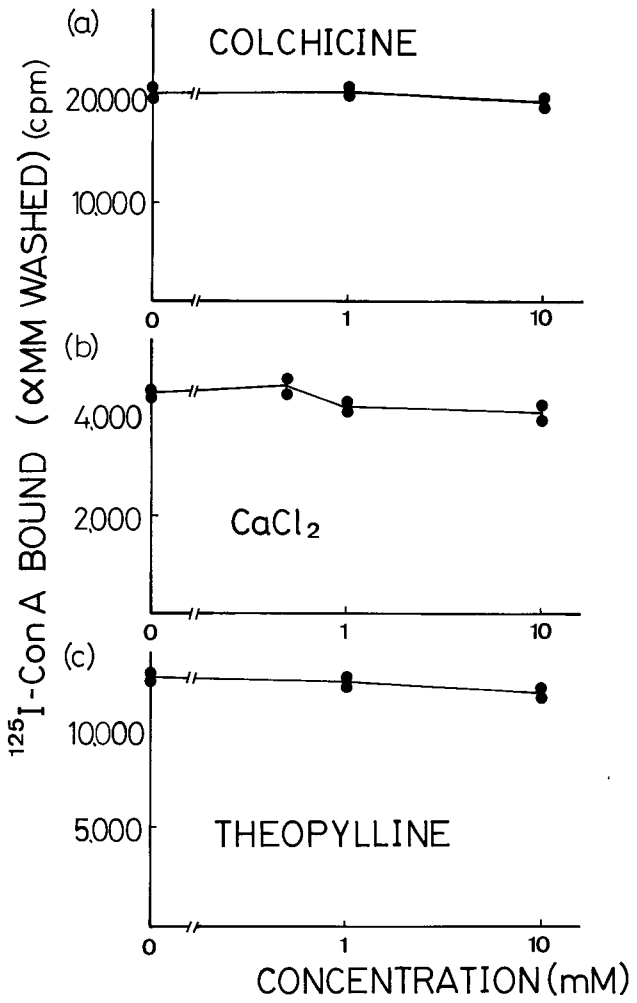

Fig. 7 Effects of colchicine (a), calcium ion (b), and theophylline (c) on pinocytosis of concanavalin A.

is used for electron microscopic experiments as described below.

As shown in Fig. 9, ferritin-con A conjugates were found in coated pits and were incorporated in small membrane vesicles present within the cells. But, quantitative study using ferritin-con A conjugates seems to be quite tedious. Therefore, we attempted the other staining method for incorporated con A by peroxidase as described by Bernhard and Avrameas (4). Since peroxidase is a glycoprotein having high affinity to con A, the site of con A binding can be visualized by osmium staining of peroxdatic polymerized products of DAB (diaminobenzidine).

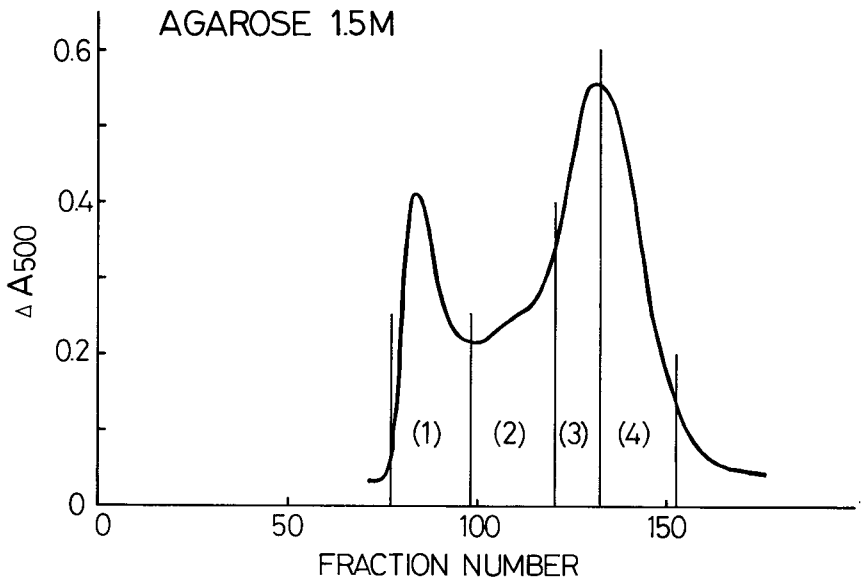

Fig. 8 Fractitonation of ferritin-concanavalin A conjugates by gel filtration. Numbers under the elution curve represent combined fractions which were used for incorporation studies and electron microscopy.

By this method, we can easily find pinocytotic vesicles containing con A in control cells which were incubated with the lectin for 30 min at 30°C, whereas no such vesicles with internalized lectin could be detected in FCCP-treated cells incubated under the conditions described in "Materials and Methods" (Fig. 10). Thus, about 40% of con A binding which is increased from 0°C control during incubation with the lectin in the presence of FCCP (Fig. 5) could be attributed to non-specific binding of the lectin to the cell surface. Degree of non-specific binding were varied from experiments to experiments when different preparations of iodinated lectin were used, therefore, partial denaturation of the lectin during iodination process seems to be responsible, at least in part, for these non-specific binding in pinocytotic measurements.

From the results reported in this paper, it can be concluded that rate of pinocytosis in Ehrlich ascites tumor cells can be quantitatively determined by measuring α-methylmannoside-resistant binding of iodinated con A provided that appropriate control experiments were performed. Pinocytotic process of the tumor cell is regulated with intracellular ATP by an unknown mechanism. Although similar ATP-dependence was found for HVJ-induced fusion of the same cells, these membrane-related processes seems to be regulated differently, because the cell fusion reaction is sensitive but pinocytosis is insensitive to cytochalasin D.

Table 1 *Pinocytotic incorporation of iodinated concanavalin A or gel filtrated fractions of ferritin-con A conjugates measured by α-methylmannoside washing method*

Incubation \ Bound (cpm)	con A	fraction 2	fraction 3	fraction 4
0°C, 5 min	1,030	720	150	170
	1;130	1,200	150	180
30°C, 30 min	8,390	5,000	1,760	1,170
	8,230	5,360	1,610	1,130

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FIGURE LEGENDS

Fig. 9 Pinocytosis of ferritin-labelled concanavalin A observed by electron microscopy.

1. Ehrlich tumor cell incubated at 30°C for 30 min with ferritin alone with the same ferritin concentration employed for ferritin-con A. No pinocytosis of ferritin was observed, indicating that con A-dependent binding is important under the condition employed. × 24,000.

2. The tumor cells were incubated with ferritin-con A for 30 min at 30°C, and then washed with α-methylmannoside. Arrow shows coated pit with ferritin label. × 50,000.

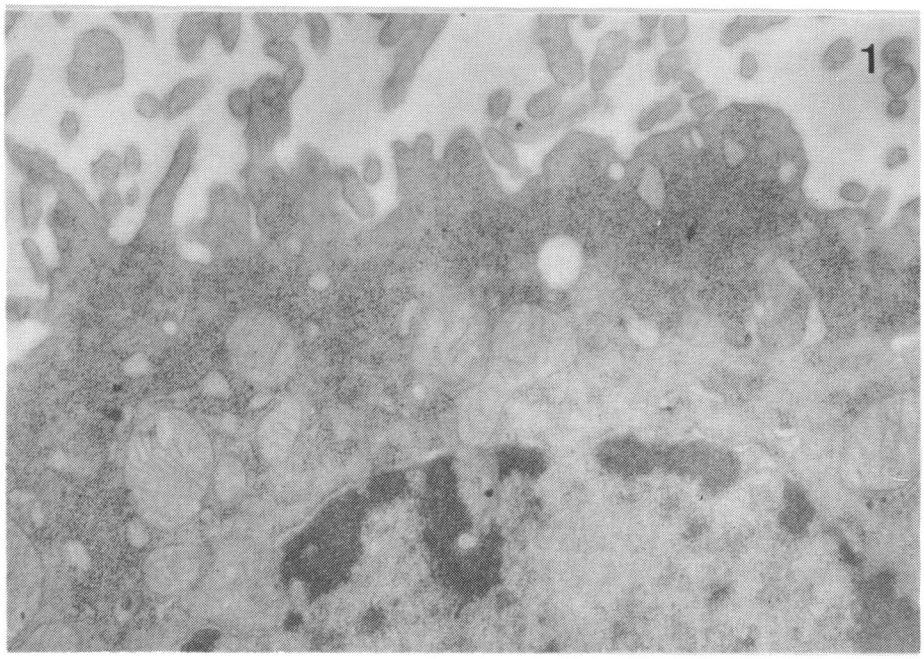
3. The same as (2), but washed with BSS. Many membrane vesicles containing ferritin label are observable. × 60,000.

Fig. 10 Pinocytosis of concanavalin A observed by peroxidase staining.

A. Pinocytosis of con A stained by peroxidase-DAB. Washed with α-methylmannoside. × 20,000.

B. The same as (A). × 12,000.

C. FCCP-preincubated cells, incubated and stained as (A). BSS washed. × 18,000.



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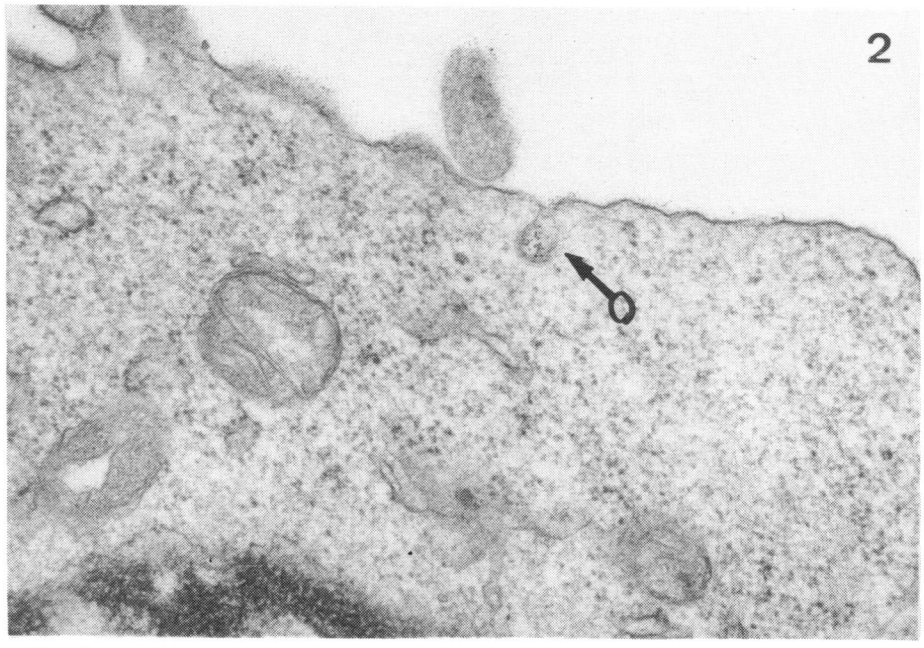

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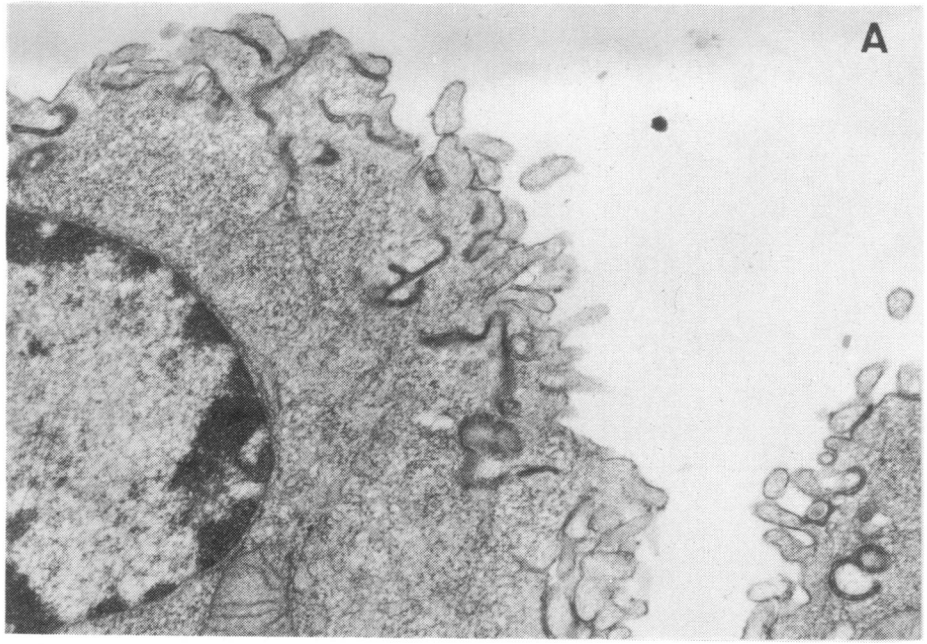

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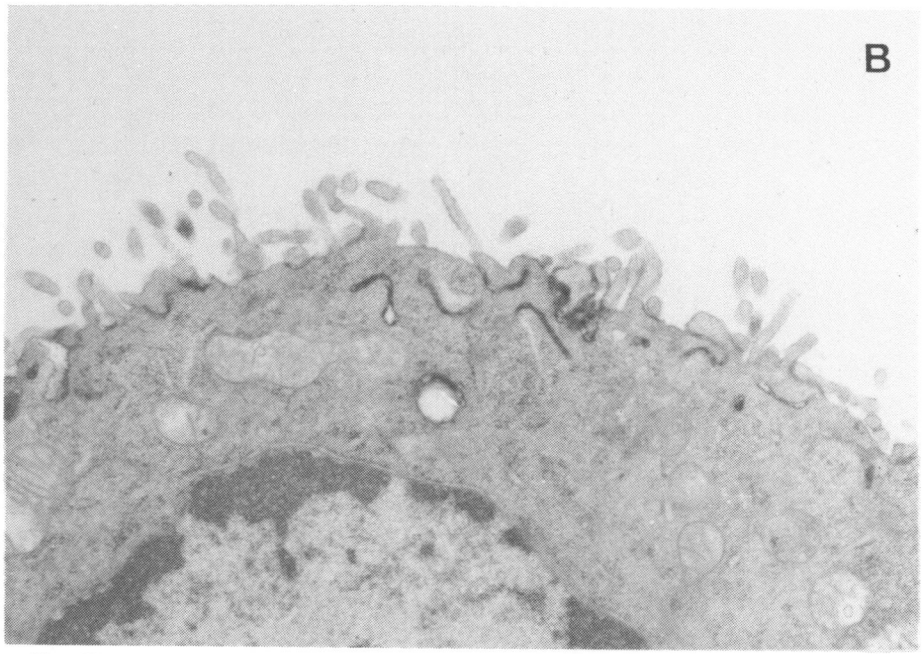

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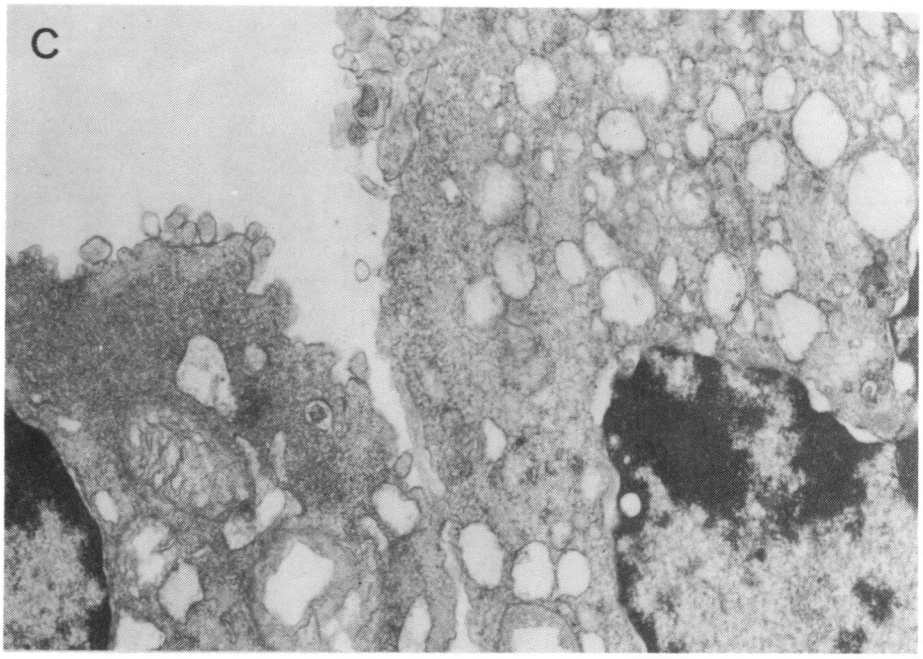

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